Standard Operating Procedure
(SOP V.1.0)

Procedure for MTBC LAMP

DNA Extraction,
Amplification and Detection of results

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1 Abbreviations

LAMP	Loop-mediated isothermal amplification
DNA	Deoxyribunucleic acid (genetic material)
PCR	Polymerase chain reaction
MTBC	Mycobacterium Tuberculosis Complex

2 Background

2.1 Introduction

Loop-mediated isothermal amplification (LAMP) of DNA is a novel molecular technology platform. The LAMP technology amplifies previously determined genes and can be used to detect any pathogen. LAMP may be considered as an alternative to polymerase chain reaction (PCR) for the detection of nucleic-acid sequences (DNA and/or RNA). Both methods amplify and detect DNA, but unlike traditional PCR, LAMP does not require a thermocycler or gel imaging system. Results can be recorded by real-time turbidimetry or through visual detection of fluorescence. Amplification and detection of the target nucleic-acid sequence is essentially completed in a single step, by incubating the mixture of sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature. LAMP provides high efficiency, with DNA being amplified 10^9-10^{10} times in 15 - 60 minutes. Therefore, LAMP can provide a result faster than traditional PCR, and can be performed in basic laboratories without the need for specialized infrastructure.

Like many other living organisms, mycobacteria store and reproduce their genetic information with DNA molecules. LAMP detects this DNA. If a person is infected with mycobacteria belonging to the Mycobacterium tuberculosis complex, the bacteria DNA can be detected in the untreated sputum or NALC-NaOH treated sputum. Normally, the DNA is present in very small amounts. Therefore, it is necessary to amplify or increase the amount of DNA in order to detect and identify it

2.2 LAMP technology

The LAMP technology is characterized by:

1. The use of a single polymerase enzyme to catalyse DNA amplification under isothermal conditions
2. Very high specificity that results from the use of four primers recognizing six distinct regions on the target DNA.
3. High amplification efficiency capable of producing high concentrations of amplified product in a short time, allowing for visual or automated detection of results.

Another advantage of the LAMP reaction is its robustness and tolerability to common PCR inhibitors. This allows the use of a 'PURE' device that rapidly removes impurities from the DNA sample.

The LAMP reaction begins with short single stranded molecules called oligonucleotides or primers. These are designed to bind to the target DNA sequence - in our case, regions in the DNA gyrase subunit B (gyrB) and Insertion sequence IS6110 of the MTBC genome. If mycobacteria DNA is present, one of the specially designed LAMP primers can anneal (bind or stick) to the complementary or matching DNA from the mycobacteria. This can occur because DNA is in dynamic equilibrium (constantly unfolding and refolding) when it reaches the reaction temperature. Primer binding initiates the process of DNA synthesis, whereby the *Bst* DNA polymerase enzyme generates new DNA that matches the mycobacteria DNA. As the DNA synthesis continues, some of the new DNA folds back on itself to form a "stem-loop" structure that looks like a dumbbell. This structure is the starting point for the amplification cycle of LAMP. The loops on the dumbbell structure now act as additional binding sites for primers to initiate DNA synthesis. As more loops are created, there are more starting points for DNA synthesis as the LAMP reaction continues. The creation of multiple loops of DNA, which provide a starting point for additional DNA synthesis, is one reason LAMP is much faster than traditional PCR.

The visual detection under ultraviolet light is based on the presence of calcein. Before DNA amplification, calcein contained in the reagent is in its quenched state as it is bound to manganese ions. Upon the start of DNA amplification, pyrophosphate ions bind to manganese ions and calcein is released producing fluorescence. If we see fluorescence, the LAMP result is positive; if there is no fluorescence, the result is negative.

2.3 Loopamp™ MTBC Detection Kit

The Loopamp™ MTBC Detection Kit is a qualitative *in vitro* diagnostic test for the detection of *Mycobacterium tuberculosis* complex (MTBC) DNA extracted from sputum in patients with any symptom indicative of an MTBC infection.

3 Purpose

These Standard Operating Procedures describe the materials, equipment, and procedures required to correctly and safely use the LAMP kit to diagnose tuberculosis using sputum samples.

This manual describes protocols to:

- Process sputum samples for DNA extraction.
- Use the LAMP kit for MTBC diagnosis.

4 Scope

This SOP has been developed for the training of laboratory personnel using the LAMP kit for MTBC diagnosis in clinical and research settings

5 Personnel qualifications

The LAMP kit for MTBC diagnosis can be used by laboratory technicians without any previous training in molecular methods. Knowledge and skills provided in this manual can be acquired in a short training period of less than 3 days. However, strict adherence to the procedures below is necessary to achieve reliable results

6 Cautions

(1) For in vitro diagnostic use only.

(2) This product is designed only for clinical diagnosis of MTBC from sputum samples of human origin. Do not use for other purposes.

(3) When using this product, always follow this package insert.

(4) Do not freeze the reagents.

(5) Do not use any expired reagent.

(6) Do not mix different lots.

(7) Do not replenish any reagent.

(8) Performance of the Loopamp™ MTBC Detection Kit is dependent on operator proficiency and adherence to procedural directions. Testing should be performed by properly trained personnel.

(9) Remove the required number of reaction tubes from the packaging just before use and seal the aluminium pouch immediately.

(10) Do not remove the desiccant from the aluminium pouch. High level of humidity may deteriorate the dried LAMP reagent in the reaction tubes.

(11) Exposure to heat and light might deteriorate the dMTB. Remove only the required number of reaction tubes (number of samples + number of controls) and seal any unused tubes immediately.

(12) Read the instruction manual of equipment involved incubator before use.

(13) Sputum samples pose a potential risk for infection. Take all necessary preventive measures to avoid biohazard.

(14) PC MTB and NC MTB both contain a small amount of sodium azide as preservative. Sodium azide is classified as toxic. Avoid any contact with eyes, mouth, or skin.

(15) In case of accidental contact of any reagent with eyes, mouth, or skin, immediately rinse the affected site with plenty of water and, if necessary, seek medical advice.

(16) Do not dilute or add the PC MTB to the samples. Use the PC MTB only as described in this package insert in order to avoid DNA contamination.

(17) Store the PC MTB positive control and any positive sputum samples separately from the other kit reagents.

(18) The cap of each reaction tube contains dMTB in the dried form. Do not touch the inside of the cap.

(19) Before using the reaction tubes, check carefully to see if they have any cracks or scratches. Damaged tubes might give false results and lead to DNA contamination of the incubator and work area.

(20) Do not expose reaction tubes to UV light before the end of the LAMP reaction. Prolonged exposure to UV light might damage the tubes and lead to false results.

(21) When a UV lamp is used for visual fluorescence judgment, do not stare directly at UV light. Since UV light is harmful to the eyes, even watching for a short period would irritate eyes and cause symptoms similar to conjunctivitis. Use a glass screen or wear goggles or a protective eye mask whenever looking directly at the UV lamp.

(22) Refer to the manual of the incubator. When the HumaLoop T or the real-time turbidimeter HumaTurb are used, be careful in removing the reaction tube from the incubator to avoid burns.

7 LAMP MTBC Assay with DNA extracted by the PURE method

The LAMP kit for MTBC diagnosis can be used with DNA extracted from sputum samples. This SOP presents the use of the PURE method which has been evaluated with patient sputum samples and NALC-NaOH treated sputum samples. The Loopamp™ **PURE DNA Extraction Kit** is a specially designed series of interlocking plastic components which provide a closed system for sample processing and direct dispensing of extracted DNA to Reaction tubes.

7.1 Sample collection and storage

The technique uses 60µL of sputum collected in a cup or 60µL of sputum samples treated with NALC-NaOH.

Transport the collected sputum in a cup with an airtight lid to a box at room temperature and send it to the laboratory on the day of sampling.

The NALC treated and untreated sample can be stored at +4°C for 1 week and 3 days respectively, if the analysis is delayed.

7.2 DNA extraction with PURE method

7.2.1 Equipment

HumaLoop T
Humax ITA （if available/necessary）

7.2.2 Materials

Permanent Marker
Disposable examination gloves (2 pairs per run)
0.5% Sodium hypochlorite
Pipette-60 Set
Loopamp™ PURE DNA Extraction Kit
Plastic Bag with zip

7.2.3 Procedures

Read the package insert of the Loopamp™ PURE DNA Extraction Kit and Loopamp™ MTBC Detection Kit carefully.

Read the HumaLoop T instruction manual carefully before operating this device.

Separate the sample transfer area from amplification area.

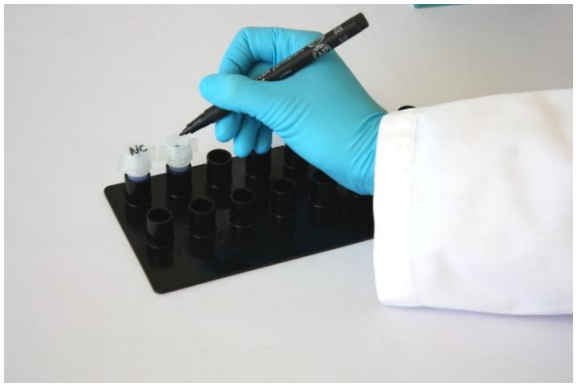
Wear two pairs of gloves!

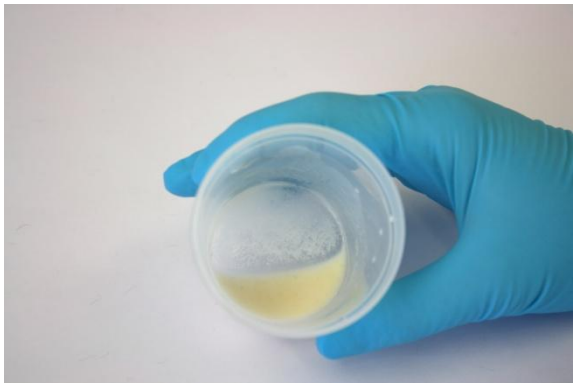
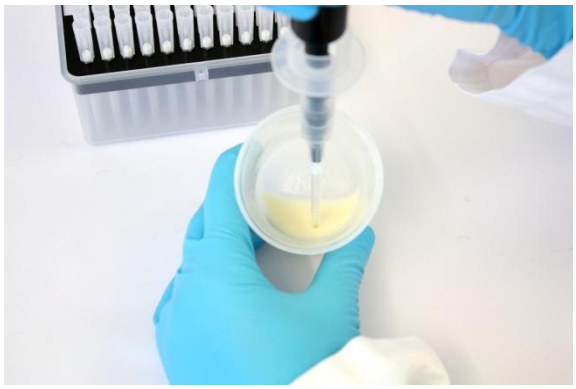
Preparation of workspace

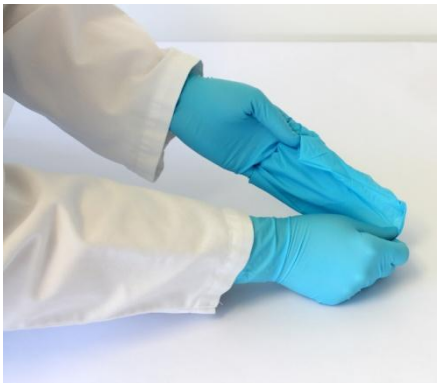
Turn on HumaLoop T	
Clean the bench with 0. 5% sodium hypochlorite (both sample transfer area and amplification area)	
Spred the solution on the surface and incubate for 5 minutes	
Wipe the surface with dry paper, then wipe again with water to remove remaining sodium hypochlorite	

All required materials must be placed into the cleaned area	

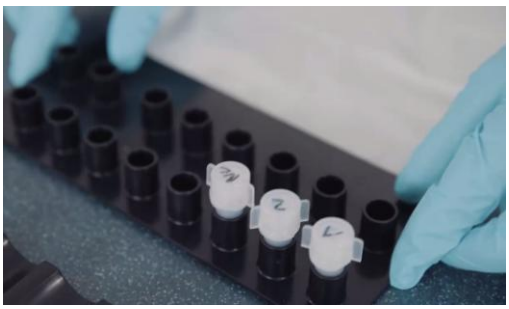
Sputum transfer

Start working at amplification area!	
Remove a sufficient number of Heating Tubes (Number of samples plus one for Negative Contol) from the aluminium pouch by pouring. Do not grab into the bag! Place them into Heating Tube stand.	
Label the lids of the Heating Tubes	
Transfer the Heating Tube stand to sample transfer area	

Open lid of the Heating Tube and place the lid on the tube. Tilt the sputum container diagonally. The sputum must be collected at one spot at the side of the container.	
Confirm the 1st and 2nd Stop of the plunger of Pipette 60-Set	
Slowly aspirate 60µL of sputum (> 5sec). Use the most purulent portion of sputum, whenever possible. Check if the volume is within the indicated range. In case of sputum strands, rub the pipette tip against the bottom of the cup to cut the strands.	

Transfer 60µL of sputum into the respective Heating Tube. Beware of sputum strands! Wash the pipette tip once with the Heating Tube solution, then check if the sputum has been release completely into the solution. Transfer 60µL of NC into the respective Heating Tube.	
Mix the Heating Tubes 3-5 times. Flick the Tube to collect the solution at the bottom of the tube.	
Discard the first pair of gloves	
Transfer the Heating Tube stand to the amplification area	

PURE Treatment

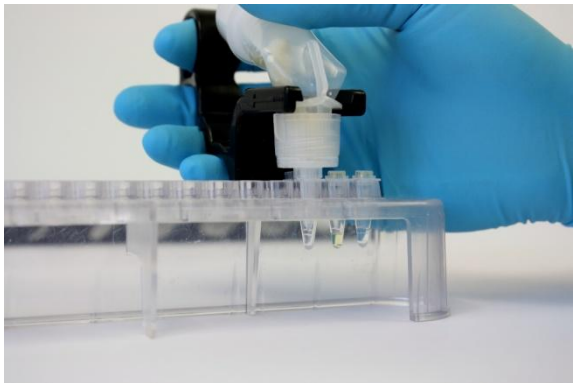
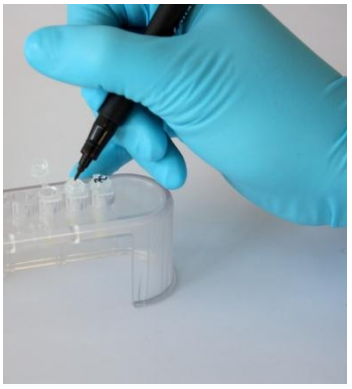
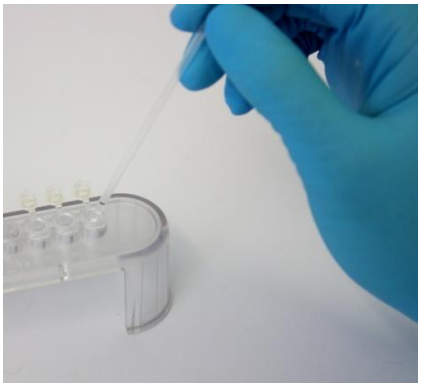
Verify that the Heating block temperature has reached 90°C.	
Load the Heating Tubes into the heating block of HumaLoop T and close the lid. Press the Start/Stop button, to start 5 minutes of incubation.	
Remove the Heating Tubes immediately after the incubation has finished. This is indicated by an audible signal. Let them cool down for 2 minutes, then mix again 3-5 times.	

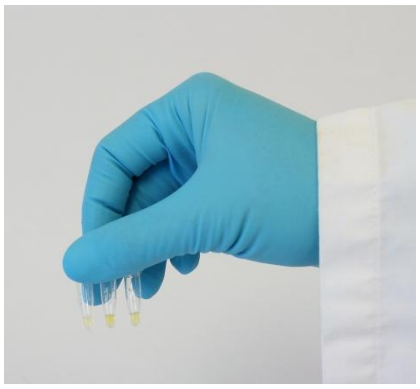
Tap the Adsorbent Tubes to loosen the white powder. Remove the cap of the Adsorbent Tube. DO NOT discard the cap! You can place it into the Heating Tube stand.	
Connect the Heating Tube to the Adsorbent Tube by screwing to release the solution into the Adsorbent Tube.	
Mix thoroughly! Powder remains, as well as pink or blue color indicate improper mixing. In this case, continue shaking and place them on the adsorbent tube tray.	

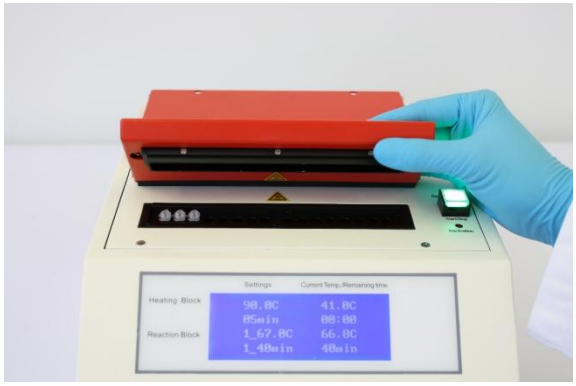
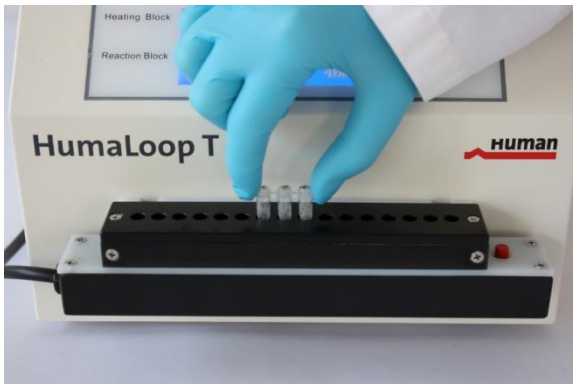
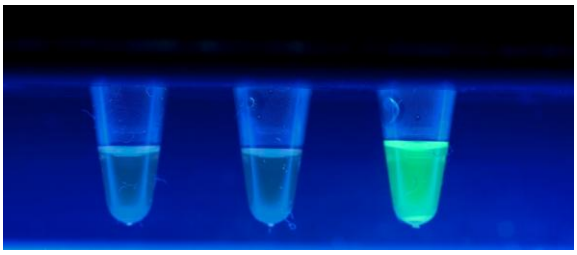
Attach the cap to the nozzle of the Injection Cap. Avoid touching the nozzle!	
Hold the Adsorbent Tube upside-down and connect the Injection Cap by pushing once and then screwing.	

7.3 DNA amplification by MTBC Detection Kit

Place a sufficient amount of Reaction Tubes into the reaction tube stand. (One Reaction Tube per Adsorbent Tube plus one for the Positive Control). Use scissors for the transfer of the tubes and place them on the rack. After removing the Reaction Tubes close the aluminum pouch tightly!	

Flick the tube to collect the solution at the bottom of the tube. Place it into the squeezing tool.	
Remove the cap. Insert the complete nozzle into the Reaction Tube. Squeeze the Adsorbent Tube and dispense 30µL of DNA directly into the Reaction Tube. The liquid level should be within the two lines on the Reaction Tube. After that, re-connect the cap again to avoid touching the nozzle.	
Close the lid of the Reaction Tube and mark it. Repeat the last five steps from attaching the cap to the nozzleto closing the lid of the Reaction Tubes for all samples and theNC.	
Dispense 30µL of PC into a Reaction Tube. Close the cap and mark it respectively. Use the provided dropper for the transfer (The black line on the dropper shows 30µL). Close the PC immediately.	

Reconstitute the dried reagent in the cap by inverting the tube. All liquid must be in the cap. Make sure that incubate upside-down for 2 minutes at room temperature. As an alternative, you could also use the HuMax ITA.	
Invert the reaction tubes 5 times to mix the content. Make sure that all liquid is in the bottom of the tube at the end.	
Make sure that the temperature of the reaction block has reached 67°C.	

Place the tubes into the reaction block and close the lid. Press the Start/Stop button. The entire amplification takes 45 minutes including the enzyme inactivation at 80°C for 5 minutes.	
After the audible signal indicates that the amplification is finished, take the Reaction Tubes out of the reaction block and put them into the fluorescence detection unit. Turn on the UV Light by pushing the red button.	
Confirm the validity of the run: PC -> green fluorescence; brighter than NC NC -> no fluorescence Positive sample -> green fluorescence Negative sample -> no fluorescence	
Document the results. Then discard the Reaction Tubes according to your local regulations. The plastic bag with zip is reccmmended to reduce the risk of contamination by leaking the LAMP product. Never do autoclave processing! Never open the Reaction Tubes after amplification!